IMPROVED PEPTIDE IDENTIFICATION AND PROTEIN COVERAGE FOR PROTEOMICS SAMPLE USING ALTERNATIVE 2D-HPLC (MS/MS) APPROACHES

Waters

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OVERVIEW

- Three off-line 2D-LC setups were compared including SCX-RP and two alternative systems: high/low pH RP-RP, and HILIC-RP.
- RP-RP (high/low pH) 2D-LC system provides the greatest number of identified peptides/proteins.
- Two methods of tandem mass spectrometry identification of peptides/proteins were compared: DDA, and a novel alternate scanning MS^E

INTRODUCTION

LC-MS methods are used for proteome research for disease markers and/or drug targets discovery. Due to the complexity of proteome samples and the wide protein dynamic range the analysis is difficult. An efficient LC separation and MS/MS analysis is required to enable the detection of lower-abundant proteins of interest in mixtures. One way to address the complexity of these samples is the application of multidimensional separation techniques like 2D-HPLC.

Currently the most common 2D-HPLC approach for separation of peptides is a combination of strong cation-exchange (SCX) and reversed-phase (RP) HPLC.

In this work we applied two alternative strategies for 2D-HPLC separation. First uses a combination of two RP-RP modes (using different pH), the second employs hydrophilic interaction chromatography (HILIC) and RP-LC. All 2D-HPLC methods were run in off-line mode using a 17 protein digests containing ~1500 peptides.

A novel alternate scanning mode for MS/MS using alternate scans at low and elevated energy has been used and compared to a traditional DDA methods.

EXPERIMENTAL

Sample Preparation

The standard proteins (Sigma) were reduced, alkylated and digested with modified porcine Trypsin (Promega) according to a standard protocol and then mixed in dynamic range 0.03-10 pmol/uL. ~8 Fractions (5 minutes) were collected in each mode (SXC, HILIC, RP high pH), volume was reduced by evaporation to the volume of original sample injected on 1st D LC (no concentration prior to 2nd D LC) and analyzed by LC-MS/MS.

HPLC Columns for fractionation

PolySULFOETHYL Aspartamide SCX 200Å, 5um 1x150mm (PolyLC Inc.)
Atlantis HILIC, 5um 1x150mm (Waters)
RP BEH-C18, 3.5um 1x150mm (Waters)

LC-MS Conditions

Tryptic peptides were analyzed by LC-MS/MS on Q-Tof Ultima (Waters). ProteinLynx Global Server V2.2 software was used for the data analysis.

RP column:	NanoEase Atlantis dC18, 3um 0.3x150mm (Waters)
RP flow:	5 ul/min
Solvent A:	0.1% Formic Acid in water
Solvent B:	0.1% Formic Acid in 80% ACN
Temperature:	40°C
Gradient:	0-45 min 0-56% B; 1% ACN/min

RESULTS AND DISCUSSION

A. SCX mode, pH 2.6

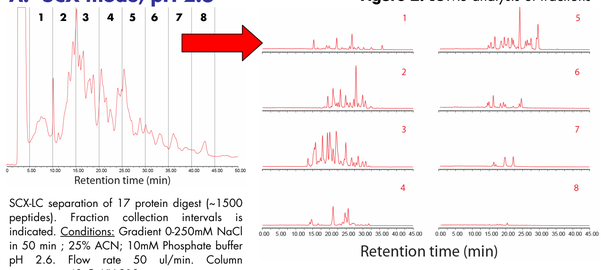

Figure 2: LC-MS analysis of fractions

SCX-LC separation of 17 protein digest (~1500 peptides). Fraction collection intervals is indicated. Conditions: Gradient 0-250mM NaCl in 50 min ; 25% ACN; 10mM Phosphate buffer pH 2.6. Flow rate 50 ul/min. Column temperature 40°C. UV 210nm.

B. HILIC mode, pH 4.5

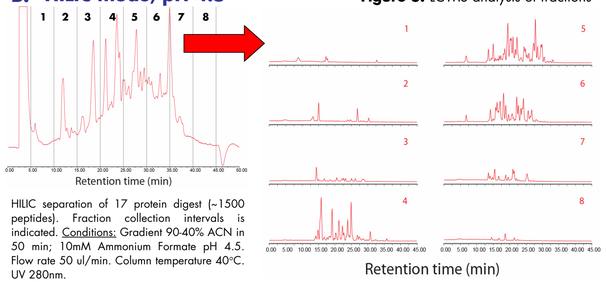

Figure 3: LC-MS analysis of fractions

HILIC separation of 17 protein digest (~1500 peptides). Fraction collection intervals is indicated. Conditions: Gradient 90-40% ACN in 50 min; 10mM Ammonium Formate pH 4.5. Flow rate 50 ul/min. Column temperature 40°C. UV 280nm.

C. RP high pH mode, pH 10.0

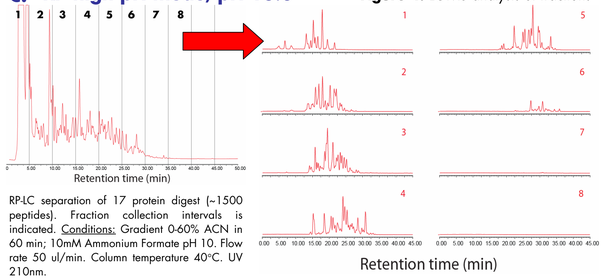

Figure 4: LC-MS analysis of fractions

RP-LC separation of 17 protein digest (~1500 peptides). Fraction collection intervals is indicated. Conditions: Gradient 0-60% ACN in 60 min; 10mM Ammonium Formate pH 10. Flow rate 50 ul/min. Column temperature 40°C. UV 210nm.

Protein Expression Analysis vs. DDA - MS/MS acquisition

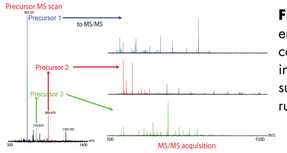

Figure 5: Data Directed Analysis (DDA). DDA employs an MS survey scan to identify abundant components (precursors) and then select these individual precursors for MS/MS fragmentation. The survey/analysis cycle is repeated throughout the run.

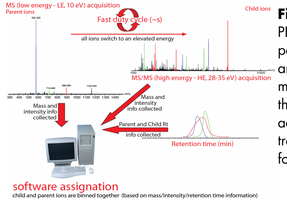

Figure 6: Protein Expression Analysis (MS^E). PE Analysis utilizes a fast cycle a low and elevated potential to collect parent and child ions. The child and parent ions are binned together via a accurate mass/intensity/retention time information. Data are then deconvoluted via software. This mode of data acquisition has an increased duty cycle over traditional DDA methods as well as a typical 5-50 fold increase in sensitivity.

Protein Expression Results

Proteins	pmol/ul	max. number of peptides *	1D LC-MS Number of peptides	SCX	HILIC	RP high pH
b-Galactosidase (E.coli)	0.03	31	-	-	-	4
b-2 Microglobulin (human)	0.05	7	-	-	-	-
a-2 Macroglobulin (human)	0.1	62	28	13	18	26
b-Lactoglobulin A	0.1	9	5	1	-	1
Ovalbumin	0.3	18	-	-	1	2
Alkoholdehydrogenase (yeast)	0.5	30	16	12	11	16
Enolase (yeast)	0.5	30	18	11	15	15
Hemoglobin (bovine)	0.5	24	8	4	4	10
Phosphorylase b (rabbit)	0.5	82	35	17	15	12
Peroxidase (horseradish)	0.5	11	-	-	-	-
Glyceraldehyd-3-phosphate dehydrogenase (rabbit)	0.5	24	3	3	4	7
Carboxypeptidase A (bovine)	0.75	12	6	-	4	6
Catalase (bovine liver)	1.5	36	27	18	23	25
Ubiquitin (bovine)	2	8	4	3	1	2
Transferrin (bovine)	2	54	38	24	27	38
a-1 Acid glycoprotein (bovine)	7.5	14	11	7	2	8
BSA	10	60	49	33	39	49
	Total:	512	248	146	164	221

Table 1: Off-line 2D LC-MS^E results for SCX, HILIC and RP high pH analysis of 17 protein digest (~1500 peptides).

* Maximum number of peptides represent peptides identified in a separate analysis of 2 pmol protein digest injection.

DDA Results

Proteins	pmol/ul	1D LC-MS Number of peptides	SCX	HILIC	RP high pH
b-Galactosidase (E.coli)	0.03	-	1	-	-
b-2 Microglobulin (human)	0.05	-	-	-	-
a-2 Macroglobulin (human)	0.1	5	4	6	9
b-Lactoglobulin A	0.1	1	3	1	3
Ovalbumin	0.3	1	1	1	3
Alkoholdehydrogenase (yeast)	0.5	9	10	6	12
Enolase (yeast)	0.5	9	6	5	8
Hemoglobin (bovine)	0.5	2	4	1	4
Phosphorylase b (rabbit)	0.5	10	6	6	12
Peroxidase (horseradish)	0.5	1	1	1	2
Glyceraldehyd-3-phosphate dehydrogenase (rabbit)	0.5	-	2	1	2
Carboxypeptidase A (bovine)	0.75	-	1	-	2
Catalase (bovine liver)	1.5	15	16	17	18
Ubiquitin (bovine)	2	1	2	1	3
Transferrin (bovine)	2	21	13	13	24
a-1 Acid glycoprotein (bovine)	7.5	7	5	4	8
BSA	10	43	21	24	36
	Total:	125	96	87	146

Table 2: Off-line 2D LC-MS/MS (Data Directed Analysis) results for SCX, HILIC and RP high pH modes.

CONCLUSIONS

- A novel methods for 2D-HPLC separation of proteomic samples have been developed.
- A proposed high/low RP-RP 2D-HPLC approach allowed for a significantly increased number of identified peptides compared to HILIC-RP and SCX-RP modes.
- The advantages of RP-RP-HPLC are high peak capacity, high peptide recovery, and the use of salt free mobile phases.
- The performance of high/low pH RP-RP-HPLC compares well to current state-of-the-art SCX-RP-HPLC results and it could be used as an alternative to SCX-RP-HPLC.
- Novel MS/MS^E method doubled the number of identified peptides and provided a significantly better confidence for protein identification in complex sample compared to a traditional DDA mode.
- Novel MS^E method represents a promising tool for proteomic research.